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ORIGINAL ARTICLE

Basic Study

Isolation and diagnosis of *Helicobacter pylori* by a new method: Microcapillary culture

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Abstract

AIM: To investigate the performance of the micro-capillary culture method (MCM) in *Helicobacter pylori* (*H. pylori*) isolation and diagnosis.

METHODS: Microcapillary culture (MC), classical culture (CC), rapid urease (CLO) test, and histo-pathologic examination (HE) were performed with biopsy samples. Homogenized biopsy samples were loaded into capillary tubes and incubated for 48 h at 37 ℃ without providing a microaerophilic environment. Additionally, three or four loops of the homogenized sample were inoculated in a ready-to-use selective medium (Becton Dickinson, Helicobacter Agar, Modified) specific for the isolation of *H. pylori* and incubated at 37 ℃ in a microaerophilic atmosphere provided by CampyGen (Becton Dickinson, GasPack). Bacteria reproducing in microcapillary tubes were evaluated in an inverted microscope and also were evaluated after performing a CC with the content. Results obtained by CC, CLO test, and HE were compared with those of MC. The diagnostic performances of the methods used in this study were evaluated for specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), and CI.

RESULTS: *H. pylori* was found positive by CLO test + HE and/or CC culture in 26 patient antrum and corpus biopsy samples. In 25 (25/26) patient biopsy samples, *H. pylori* was isolated by MCM, whereas in only 14 (14/26) patient biopsy samples, *H. pylori* was isolated

by CC. CLO test and HE were found positive in 17 (17/26) patient biopsy samples. Comparing the results of the isolation of *H. pylori* by MCM, CC, CLO test, and HE, the sensitivity of the MCM was found as 96%, the specificity as 80%, the PPV as 83%, the NPV as 95%, and the 95%CI as 0.76 (χ^2 = 31.51, P < 0.01) whereas the sensitivity of the CC was found as 54% (χ^2 = 19.15, P < 0.01), and the sensitivity of the CLO test and HE were found as 65% (χ^2 = 25.26, P < 0.01).

CONCLUSION: This new microcapillary cultivation method for *H. pylori* has high diagnostic sensitivity compared with CC, HE, and CLO tests.

Key words: Microcapillary culture; Classical culture; New method; Isolation of *Helicobacter pylori*; Comparison

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Core tip: Nowadays, isolation of *Helicobacter pylori* (*H. pylori*) from gastric tissues by using classical culture is accepted as the gold standard, according to Maastricht criteria. Additionally, surveillance of increased or decreased minimal inhibitory concentration values of antimicrobial substances, according to applied treatment policy and geographical regions, may only be possible with the cultivation of bacteria. On the other hand, the diagnostic sensitivity of classical culture is low due to problems in standardization of the medium in order to provide microaerophylic conditions. In this study, for the first time, we report the high diagnostic sensitivity of the microcapillary culture method for diagnosis of *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic, and spiral-shaped bacterium that affects more than half of human population worldwide and is especially more populated in developing countries. Various difficulties during its diagnosis, evolution of drug resistant strains, and absence of an effective vaccine have resulted in the global spread of *H. pylori*. *H. pylori* causes important health disorders such as chronic gastritis, peptic ulcer, gastric cancer, and gastric lymphoma[1-6].

There are now several invasive methods for the clinical diagnosis of *H. pylori*, such as histopathologic examination (HE), rapid urea (CLO) test, and classical culture (CC) as well as noninvasive methods such as serology, 13C-urea breath test, and the stool antigen test[7-9]. Of these methods, the cultivation of *H. pylori* from gastric biopsies taken from patients is the most specific and most sensitive. However, cultivation of *H. pylori* requires specific agar and special atmospheric conditions, which hinder its routine use as a diagnostic method. Whereas, histopathologic detection of *H. pylori* in samples obtained from the stomach, corpus, and antrum has been reported to be a more sensitive method than both the CLO test and CC[10]. However, histopathologic detection requires expert pathologists for the accurate examination of the samples.

Although a CLO test is economic, fast, and has more than 90% specificity, this method would return negative results in cases having an actively bleeding ulcer and intestinal metaplasia and in patients being treated with proton pump inhibitor (PPI) medications. In addition, this method may also return false-positive results when urease-positive bacteria are present in the specimen. For these reasons, the CLO test has lower sensitivity[11-13]. Although the ^{13}C-urea breath test shows high sensitivity for adults, its sensitivity is lower for children and patients at the beginning of the disease. Additionally, it requires the use of gas chromatography and mass spectroscopy, which increase its cost. Serological tests relying on antibody detection have 85% sensitivity and 80% specificity but are considered insufficient for follow-up of the treatment[8-14].

The *H. pylori* stool antigen test, which is a practicable serological method for detection of the antigen before and after treatment, includes the risk of false negative results for patients that use PPI medications and bismuth derivatives[9-14]. Despite the fact that several invasive and noninvasive methods exist for the diagnosis of *H. pylori*, none of these have been accepted as a gold standard[15,16].

Since the primary target for the clinical diagnosis of *H. pylori* is the treatment of patients, following up the development of resistant bacteria strains against conventional antibiotic therapy is important, both for preventing cases that are not responding to current treatment and preventing relapses of the diseases. Surveillance studies to investigate the antimicrobial resistance of *H. pylori* can be performed by isolating the bacteria[17]. However, the disadvantages of the present culture methods do not allow one to easily perform this type of research[12,15]. Therefore, it is crucial to develop new methods or optimize common methods so that they are free from these disadvantages.

In our previous study, it was demonstrated that one microaerophilic microorganism, *Leishmania* promastigotes, was rapidly grown by microcapillary culture method (MCM), independent of the number of parasites[18,19]. This method depends on the transfer of a small number of samples into a hematocrit capillary and the detection of microaerophilic microorganisms, which are rapidly grown in this environment. We employ

observations of the specific motility characteristics of these microorganisms using a microscope[18,19]. Nowadays, MCM is primarily used for the diagnosis of Leishmaniasis, and it has been found that this method is more specific, sensitive, and economical than conventional culture methods.

Since the MCM may yield microaerophilic conditions that are essential for the survival of *H. pylori*, we suggest that MCM also can be used for the diagnosis and sufficient production of *H. pylori*. Furthermore, we maintain that growth of the bacteria *via* MCM could allow for the detection of bacteria in samples that include only a low number of bacteria and could decrease contamination risks that arise from the use of selective media for *H. pylori* cultivation. However, there have been no studies in the literature demonstrating the growth of *H. pylori via* MCM. Therefore, as a proof of concept, we investigate for the first time the efficacy of MCM in diagnosis and isolation of *H. pylori* and compare its diagnostic performance with classical culture, histopathology, and rapid urea tests.

MATERIALS AND METHODS

Patients and control group

This diagnostic test study intended for the production of *H. pylori* using MCM was conducted with dyspeptic patients having an endoscopy indication. These patients were admitted to the endoscopy unit of the Gastroenterology Department of Cerrahpasa School of Medicine between September and December 2012. The patient and control groups consisted of participants that satisfied the following criteria: no previous infection with *H. pylori*; no history of gastric surgery; not having taken *H. pylori* eradication treatment, antibiotic, or antisecretory drug within the last two weeks; not having taken bismuth salts; and without bleeding and clotting disorders. The patient group was formed from patients in whom biopsy samples were found positive by at least two diagnostic tests, such as histopathology and rapid urease test and/or CC. The control group was formed of subjects whose biopsy samples were found negative for *H. pylori* by histopathology and rapid urease test, and/or CC. The present study has been approved by the Istanbul University Cerrahpasa Faculty of Medicine Ethical Committee with the document number: 4548.

Sample collection

Three antrum and corpus biopsies were taken from the patients, one of which was sent to the pathology laboratory. The remaining two biopsies were placed immediately in tubes containing 20% brucella broth with glucose and were transported to two different laboratories in ice (4 ℃). CC and rapid urease tests were performed with biopsies transported to the Microbiology Laboratory of Cerrahpasa Medical Faculty. The other biopsy samples were transported to the Bioengineering Department of Yildiz Technical University, where they were treated for microcapillary culture. The cultures were independently performed in these two laboratories, and the results were maintained secret until all the subjects had been screened.

Classical culture

The classical culture was performed in the Microbiology Laboratory of Cerrahpasa Medical Faculty. Biopsy samples carried in tubes containing 500 μL brucella broth (Biolife) with 20% glucose were homogenized with a glass rod in the same tube in a laminar flow hood. Three or four loops of this homogenized sample were inoculated in a ready-to-use selective medium (Becton Dickinson, Helicobacter Agar, Modified) specific for the isolation of *H. pylori* and containing Columbia agar, 10% defibrinated horse blood, 10 mg/L vancomycin, 10 mg/L amphotericin B, 5 mg/L cefsulodin, and 5 mg/L trimethroprim and incubated at 37 ℃. The microaerophilic atmosphere was provided by CampyGen (BD GasPack). After an incubation period of 72 h, the colony morphology of bacteria and their Gram-staining characteristics were studied. Convex, semitransparent, 1-2 mm diameter colonies with positive catalase, urease, and oxidase activity were evaluated as *H. pylori*. Part of these colonies were inoculated into brucella broth containing 20% glycerol and stored at -80 ℃[14,15].

Microcapillary culture

The microcapillary culture was performed in laboratories of the Bioengineering Department of Yildiz Technical University. Microcapillary tubes (ISOLAB) were put in a solution of potassium chromate for 1 h then rinsed with pure water. They were sterilized in a Pasteur oven at 180 ℃ for 1 h. Biopsy samples carried in dry ice, in tubes containing 500 μL brucella broth (Biolife) with 20% glucose, were homogenized with the same method used in CC. In each microcapillary tube, 60 μL of the homogenized sample were transferred, and the ends of the tubes were closed with sterile silicone. The microcapillary tubes were incubated at 37 ℃ for 48 h, and no CO_2 atmosphere provider was used. After the incubation period, the end of three capillary tubes were broken in a laminar flow in sterile conditions, and the content was transfered to a ready-to-use selective agar specific for the isolation of *H. pylori* (Becton Dickinson, Helicobacter Agar, Modified) and containing Columbia agar, 10% defibrinated horse blood, 10 mg/L vancomycin, 10 mg/L amphotericin B, 5 mg/L cefsulodin, and 5 mg/L trimethroprim. The media were incubated at 37 ℃ in a microaerophilic atmosphere provided by CampyGen. After the incubation period, convex, semitransparent, 1-2 diameter colonies with positive catalase, urease, and oxidase activity were evaluated as *H. pylori*. Additionally, a polymerase chain reaction (PCR)

Figure 1 Microscopic view of *Helicobacter pylori* cultivated in a microcapillary tube.

performed with the DNA of these strains was used for the confirmation. The standard strain of *H. pylori* 26695 (ATCC 700392) was used as a control.

Invert microscopy

After an incubation period of 48 h, microcapillary tubes containing homogenized biopsy samples were removed from the incubator, and their surfaces were cleaned by dry linen. The tube was placed on the microscope table, and the cylindrical surface of the capillary tube was found with a 10 × ocular and 10 × objective (Figure 1). Then using 20 × and 40 × objective, the presence of *H. pylori* was studied through the tube. Spiral-shaped, flagellated microorganisms with characteristic movement of *H. pylori* were interpreted as *H. pylori*.

PCR

H. pylori strains isolated by MCM were confirmed by PCR. For the isolation of the DNA, transparent colonies (1-2 mm in diameter) were collected in 1.5 mL Eppendorf tubes containing 1 mL PBS. The Eppendorfs were centrifuged at 3000 *g* for 15 min. DNA extraction was performed with a high pure PCR template preparation kit (Roche Diagnostics GmBH, Germany), according to the manufacturer's instruction. Hp1 (Forward; 5'CTG GAG AGA CTA AGC CCT CC3') and Hp2 (Reverse; 5'ATT ACT GAC GCT GAT TGT GC3') primers belonging to the 110 bp of 16srRNA gen region of *H. pylori* were amplified in a T100 thermal cycler (Biorad, United States)[20]. The PCR content was prepared with a PCR Master Mix kit (Fermentas, ThermoFisher Scientific, United States) according to the manufacturer's instruction. In a total of 50 μL of reaction volume, 25 μL PCR Master Mix, 3 μL forward primer, 3 μL reverse primer, 5 μL isolated DNA, and 14 μL PCR grade water were used. The reaction run of the amplification protocol were one cycle of 95 ℃ for 3 min, 38 cycles of 60 ℃ for 1 min, and 95 ℃ for 30 s; one cycle of 72 ℃ for 3 min. For the electrophoresis, 2% agarose gel was prepared with 0.6 gr agarose, 2 μL ethidium bromide and 30 mL TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA, pH 8.0). 1 μL 6 × Loading Dye (Fermentas) and 5 μL amplicon were loaded into each well. Additionally, 1 μL of 100 bp DNA ladder, positive and negative controls were loaded into the appropriate well. Then gel was run for 20 min in 55 mA and 110 V. Bands were visualized in an UV monitor gel Doc EZ System (Biorad, United States). Bands similar to that of 110 bp band of the positive control were accepted as positive.

Statistical analysis

Data were analyzed with SPSS 15.0 statistical software (Statistical Packages for Social Sciences; SPSS Inc., Chicago). Diagnostic performances of methods were calculated with specificity, sensitivity, PPV, NPV, and 95%CI.

RESULTS

H. pylori was found positive by CLO test + HE and/or CC in 26 patient antrum and corpus biopsy samples. In 25 (25/26) patient biopsy samples, *H. pylori* was isolated by MCM, whereas in only 14 (14/26) patient biopsy samples it was isolated by CC. In 17 (17/26) patient biopsy samples, CLO test and HE were found positive (Table 1).

In 25 patient biopsy samples, *H. pylori* was isolated by MCM, whereas in only 16 (16/25) of them *H. pylori* was found positive by CLO test and HE, and in only nine (9/25) patient biopsy samples *H. pylori* was isolated by CC. The presence of *H. pylori* isolated by the MCM was confirmed by at least one more method (Table 1). Comparing the results of the isolation of *H. pylori* by MCM, CC, CLO test, and HE, the sensitivity of the MCM was found as 96%, the specificity was found as 80%, the PPV was found as 83%, the NPV was found as 95%, and the κ coefficient of concordance was found as 0.76 (χ^2 = 31.51, P < 0.01) (Table 2).

H. pylori was isolated by CC in 14 (14/26) patient biopsy samples. Nine (9/14) of them were also found positive by MCM, and five (5/14) of them were found positive both by MCM, CLO test, and HE. The sensitivity of the CC was found as 54%, the specificity was found as 100%, the PPV was found as 100%, the NPV was found as 68%, and the κ coefficient of concordance was found as 0.54 (χ^2 = 19.15, P < 0.01) (Table 2).

H. pylori was found positive by CLO test and HE in a total of 17 patient biopsy samples (17/26). In 11 (11/17) of them, *H. pylori* was isolated by MCM, and in five (5/17) of them *H. pylori* was isolated both by MCM and CC. In one patient biopsy samples, *H. pylori* was not isolated by MCM and CC; it was determined only by CLO test and HE. The sensitivity of the CLO test and HE was found as 65%, the specificity was found as 100%, the PPV was found as 100%, the NPV was found as 74%, and the κ coefficient of concordance was found as 0.64 (χ^2 = 25.26, P < 0.01) (Table 2).

Table 1 *Helicobacter pylori* isolation results of 26 biopsies tested according to different diagnostic methods

Biopsies	CC	MCM	HE	CLO
1	+	+	+	+
2	+	+	-	-
3	-	+	+	+
4	-	+	+	+
5	+	+	-	-
6	+	+	+	+
7	-	+	+	+
8	-	+	+	+
9	+	+	-	-
10	-	+	+	+
11	+	+	-	-
12	-	+	+	+
13	+	+	+	+
14	+	+	+	+
15	-	+	+	+
16	-	+	+	+
17	+	+	+	+
18	+	+	-	-
19	-	+	+	+
20	+	+	-	-
21	-	+	+	+
22	+	+	-	-
23	+	+	-	-
24	-	+	+	+
25	+	+	+	+
26	-	-	+	+

CC: Classical culture; HE: Histopathologic examination; CLO: Rapid urease test; MCM: Microcapillary culture method.

Table 2 Diagnostic performance parameters of the microcapillary culture method, classical culture, histopathologic examination and rapid urease test

	H. pylori (+) reference	*H. pylori* (-) control
MCM[1]		
+	25	5
-	1	21
Total	26	26
CC[2]		
+	14	-
-	12	26
Total	26	26
HE[3] and CLO[4] test		
+	17	-
-	9	26
Total	26	26

[1]Diagnostic performance parameters of microcapillary culture method (MCM): Sensitivity: 96%, specificity: 80%, positive predictive value (PPV): 83%, negative predictive value (NPV): 95%, kappa: 0.76; [2]Diagnostic performance parameters of classical culture (CC): Sensitivity: 54%, specificity: 100%, PPV: 100%, NPV: 68%, kappa: 0.54; [3]Diagnostic performance parameters of histopathologic examination (HE): Sensitivity: 65%, specificity: 100%, PPV: 100%, NPV: 74%, kappa: 0.64; [4]Diagnostic performance parameters of rapid urease (CLO) test: Sensitivity: 65%, specificity: 100%, PPV: 100%, NPV: 74%, kappa: 0.64. *H. pylori*: *Helicobacter pylori*.

DISCUSSION

Invasive and noninvasive tests can be used for the *in vitro* diagnosis of *H. pylori*, which has a role in the pathologies of duodenal gastritis, mucosa-associated lymphoid tissue lymphoma, and gastric carsinoma[12,21,22]. The isolation of the bacteria from gastric tissues by CC as a diagnostic tool is problematic due to its low sensitivity[22]. In the CC method, the incubation conditions, optimization of the media ingredients, and contamination problems specific to the biopsy specimen can be cited as the essential problems[12,22]. Although the isolation of *H. pylori* from gastric tissues by CC was accepted as a reference method, according to Maastricht criteria, due to factors such as late reproduction of the bacteria and low diagnostic sensitivity of the method, it has not been used in the routine diagnosis[22].

Although there are difficulties with the CC, viable and active *H. pylori* strains are a requirement for several important research efforts, including cagA-EPIYA-gastric carcinoma research, the determination of antimicrobial resistance patterns, and the development of new treatment options. All of these have highlighted the importance of the culture method[17,23].

Due to the importance of *H. pylori* isolation by culture, the search for an alternative isolation method is ongoing. In this study, for the first time in the world, the MCM was used for the diagnosis of *H. pylori*, and its diagnostic performance was evaluated relative to CC, HE, and the CLO test. We found the diagnostic sensitivity of the MCM to be 96%, with a specificity of 80%. The PPV was 83%, the NPV was 95%, and the κ coefficient of concordance was 0.76. According to these results, the sensitivity of the MCM was higher than that of CC (54%), HE, and CLO tests (65%). In contrast, the specificity of MCM (80%) is lower than the CC (100%). It has been shown that the sensitivity of the CC is affected by many factors, such as the density of bacteria, contamination, atmospheric environment, the use of antibiotics or PPIs, and the content of medium[12,22]. In our study, the diagnostic sensitivity of the CC was likely subdued because of these factors. In contrast, the MCM was unaffected by these, and, additionally, it was found that it provided a more suitable microaerophilic environment that increased the growth of the bacteria by facilitating its adaptation to the environment.

In our previous study, we have shown that *Leishmania* parasites grow better in a microcapillary environment compared with the classical culture of the parasite[18]. In this case, the microculture, together with higher CO_2 concentrations, lower O_2 concentrations, and lower pH, ensures the microaerophilic environment necessary for the growth of *Leishmania* parasites. In addition, the higher parasite load helped to increase the concentration of autocrine growth factors in the medium. We believe that similar factors of the MCM are effective in achieving good *H. pylori* growth. Blakemore[24] and Wolfe *et al*[25] noted that some of the microaerophilic bacteria (named magnetic bacteria) concentrated in microcapillary tubes, due to changes

to their chemotaxis provided by the magnetic forces of intracellular iron particles. Similar factors are likely to be involved in *H. pylori* growth in microcapillary tubes.

The higher diagnostic sensitivity of MCM can be evaluated positively when considering the 11 cases in which the CLO test and HE were found positive and CC was found negative. Thus, these tissue samples were found *H. pylori* positive by the MCM, and, despite the HE and CLO test also being positive, *H. pylori* was found negative in the CC, probably because of the low density of bacteria. Although the diagnostic sensitivity of MCM is high, the specificity was lower compared with the CC. In five cases of the control group, *H. pylori* was found positive by MCM; although it should be noted that, in these cases, the positive results were also identified by a PCR method. As the molecular methods for the diagnosis of biopsy samples have yet to be fully optimized, these cases were evaluated as *H. pylori* negative, which classifies these cases as false positives, according to Maastrich diagnostic reference methods. However, these findings suggest that it might be useful to perform future investigations on patients with dyspeptic complaints and on a large series of *H. pylori* negative cases diagnosed by reference methods.

Another striking observation from this study is that, in nine biopsy samples returning a HE negative result, *H. pylori* could be isolated by CC and MCM. Many reports have referred to false negative histopathology results due to inappropiate biopsy region selection, nonhomogenous distribution of bacteria in the tissue, small amounts of bacteria, or due to the presence of a very low contrast difference between the biopsy and *H. pylori*[22]. The isolation of *H. pylori* in these samples increased the diagnostic performance of this method.

The CLO test, which is used for the diagnosis of gastritis associated with *H. pylori,* can result in false positives for several reasons: (1) contamination by other urease enzyme producing bacteria; (2) incorrect implementation of the CLO test during endoscopy; (3) temporary decrease of bacteria due to antibiotics and PPIs; and (4) inappropiate biopsy region[22]. Therefore, when used alone, the CLO test has low diagnostic performance. Lee *et al*[22] in 2013 found that the sensitivity of the CLO test made with either antrum or corpus samples was 60%-65%. When the test was performed with both antrum and corpus samples, the sensitivity reached 85%, although this was still lower than that reported for the CC method (91%). Here, the CLO test was found positive in 17 corpus and antrum samples. *H. pylori* was isolated by CC and MCM in nine samples in which the CLO test result was negative. Thus, we found that MCM outperforms the CLO test in terms of sensitivity in the laboratory diagnosis of *H. pylori*.

In conclusion, our results indicate that this new cultivation method for *H. pylori* by microculture is advantageous with regard to its high diagnostic sensitivity compared with CC, HE, and CLO tests, and because the method itself produces microaerophilic conditions which eliminate the need for a microaerophilic environment provider kit; it also has the property of occupying a very small space in incubators. Further studies, including a large number of cases with dyspeptic complaints, are needed before we can encourage the routine usage of this new cultivation method for the diagnosis of *H. pylori*.

COMMENTS

Background

The isolation of *Helicobacter pylori* (*H. pylori*) from gastric tissues by classical culture is problematic due to the need for specific atmospheric incubation conditions and optimization of the media ingredients. In this study, the authors report a new "microcapillary culture" method for the diagnosis and isolation of *H. pylori*, and they compare its diagnostic performance with classical culture, histopathology, and rapid urea test.

Research frontiers

In this study, *H. pylori* was cultivated in microcapillary tubes, and the diagnostic performance of this new cultivation method was compared with classical culture (CC), histopathologic examination (HE), and rapid urease (CLO) test. This is the first report showing that *H. pylori* can be isolated and cultivated from gastric tissues by a microculture method, which has a high diagnostic sensitivity.

Innovations and breakthroughs

In this study, the microcapillary culture method (MCM) was used for the first time in the world for the cultivation and isolation of *H. pylori* from gastric tissues. The results showed that, in microcapillary tubes, a higher CO_2 concentration, lower O_2 concentration, and lower pH ensures the microaerophilic environment necessary for the growth of *H. pylori*, and that this new method has a higher isolation sensitivity than CC, HE, and CLO test.

Applications

This study showed that *H. pylori* can be cultivated by MCM without the need of a microaerophilic environment provider kit or method. This finding provided a new cultivation method useful for the isolation and diagnosis of *H. pylori* from gastric tissues.

Peer-review

This study is concerned with a new cultivation method of *H. pylori* from gastric tissues. It states that the MCM proposed for the isolation and cultivation of *H. pylori* from gastric tissues without the need of a microaerophilic environment provider kit or method is more sensitive then CC, HE, and CLO test.

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